

Selected clones can be grown overnight in liquid culture medium such as LB broth supplemented with antibiotics. The overnight culture may subsequently be used to inoculate a larger scale culture. The cells are then grown to a desired optical density, during which the expression promoter is turned on.

After culturing the cells for several more hours, the cells can be harvested by centrifugation. The cell pellet obtained by the centrifugation can be solubilized using various agents known in the art, and the solubilized PRO polypeptide can then be purified using a metal chelating column under conditions that allow tight binding of the protein.

PRO181, PRO195, PRO200, PRO237, PRO273, PRO540, PRO322, PRO1017, PRO938, PRO162, PRO1114, PRO827 and PRO1008 were expressed in *E. coli* in a poly-His tagged form, using the following procedure. The DNA encoding the PRO polypeptide was initially amplified using selected PCR primers. The primers contained restriction enzyme sites which correspond to the restriction enzyme sites on the selected expression vector, and other useful sequences providing for efficient and reliable translation initiation, rapid purification on a metal chelation column, and proteolytic removal with enterokinase. The PCR-amplified, poly-His tagged sequences were then ligated into an expression vector, which was used to transform an *E. coli* host based on strain 52 (W3110 fuhA(tonA) lon galE rpoHts(htpRts) clpP(lacIq)). Transformants were first grown in LB containing 50 mg/ml carbenicillin at 30°C with shaking until an O.D.600 of 3-5 was reached. Cultures were then diluted 50-100 fold into CRAP media (prepared by mixing 3.57 g (NH₄)₂SO₄, 0.71 g sodium citrate·2H₂O, 1.07 g KCl, 5.36 g Difco yeast extract, 5.36 g Sheffield brycase SF in 500 mL water, as well as 110 mM MPOS, pH 7.3, 0.55% (w/v) glucose and 7 mM MgSO₄) and grown for approximately 20-30 hours at 30°C with shaking. Samples were removed to verify expression by SDS-PAGE analysis, and the bulk culture is centrifuged to pellet the cells. Cell pellets were frozen until purification and refolding.

E. coli paste from 0.5 to 1 L fermentations (6-10 g pellets) was resuspended in 10 volumes (w/v) in 7 M guanidine, 20 mM Tris, pH 8 buffer. Solid sodium sulfite and sodium tetrathionate is added to make final concentrations of 0.1M and 0.02 M, respectively, and the solution was stirred overnight at 4°C. This step results in a denatured protein with all cysteine residues blocked by sulfitolization. The solution was centrifuged at 40,000 rpm in a Beckman Ultracentrifuge for 30 min. The supernatant was diluted with 3-5 volumes of metal chelate column buffer (6 M guanidine, 20 mM Tris, pH 7.4) and filtered through 0.22 micron filters to clarify. Depending the clarified extract was loaded onto a 5 ml Qiagen Ni-NTA metal chelate column equilibrated in the metal chelate column buffer. The column was washed with additional buffer containing 50 mM imidazole (Calbiochem, Utrol grade), pH 7.4. The protein was eluted with buffer containing 250 mM imidazole. Fractions containing the desired protein were pooled and stored at 4°C. Protein concentration was estimated by its absorbance at 280 nm using the calculated extinction coefficient based on its amino acid sequence.

The proteins were refolded by diluting sample slowly into freshly prepared refolding buffer consisting of: 20 mM Tris, pH 8.6, 0.3 M NaCl, 2.5 M urea, 5 mM cysteine, 20 mM glycine and 1 mM EDTA. Refolding volumes were chosen so that the final protein concentration was between 50 to 100 micrograms/ml. The refolding solution was stirred gently at 4°C for 12-36 hours. The refolding reaction was quenched by the addition of TFA to a final concentration of 0.4% (pH of approximately 3). Before further purification of the protein, the solution was filtered through a 0.22 micron filter and acetonitrile was added to 2-10% final concentration. The refolded protein was chromatographed on a Poros R1/H reversed phase column using a

mobile buffer of 0.1% TFA with elution with a gradient of acetonitrile from 10 to 80%. Aliquots of fractions with A280 absorbance were analyzed on SDS polyacrylamide gels and fractions containing homogeneous refolded protein were pooled. Generally, the properly refolded species of most proteins are eluted at the lowest concentrations of acetonitrile since those species are the most compact with their hydrophobic interiors shielded from interaction with the reversed phase resin. Aggregated species are usually eluted at higher acetonitrile concentrations. In addition to resolving misfolded forms of proteins from the desired form, the reversed phase step also removes endotoxin from the samples.

Fractions containing the desired folded PRO proteins were pooled and the acetonitrile removed using a gentle stream of nitrogen directed at the solution. Proteins were formulated into 20 mM Hepes, pH 6.8 with 0.14 M sodium chloride and 4% mannitol by dialysis or by gel filtration using G25 Superfine (Pharmacia) resins equilibrated in the formulation buffer and sterile filtered.

Many of the PRO polypeptides described herein were successfully expressed as described above.

EXAMPLE 101: Expression of PRO Polypeptides in Mammalian Cells

This example illustrates preparation of a glycosylated form of a desired PRO polypeptide by recombinant expression in mammalian cells.

The vector, pRK5 (see EP 307,247, published March 15, 1989), is employed as the expression vector. Optionally, the PRO polypeptide-encoding DNA is ligated into pRK5 with selected restriction enzymes to allow insertion of the PRO polypeptide DNA using ligation methods such as described in Sambrook et al., *supra*. The resulting vector is called pRK5-PRO polypeptide.

In one embodiment, the selected host cells may be 293 cells. Human 293 cells (ATCC CCL 1573) are grown to confluence in tissue culture plates in medium such as DMEM supplemented with fetal calf serum and optionally, nutrient components and/or antibiotics. About 10 μ g pRK5-PRO polypeptide DNA is mixed with about 1 μ g DNA encoding the VA RNA gene [Thimmappaya et al., *Cell*, 31:543 (1982)] and dissolved in 500 μ l of 1 mM Tris-HCl, 0.1 mM EDTA, 0.227 M CaCl_2 . To this mixture is added, dropwise, 500 μ l of 50 mM HEPES (pH 7.35), 280 mM NaCl, 1.5 mM NaPO_4 , and a precipitate is allowed to form for 10 minutes at 25°C. The precipitate is suspended and added to the 293 cells and allowed to settle for about four hours at 37°C. The culture medium is aspirated off and 2 ml of 20% glycerol in PBS is added for 30 seconds. The 293 cells are then washed with serum free medium, fresh medium is added and the cells are incubated for about 5 days.

Approximately 24 hours after the transfections, the culture medium is removed and replaced with culture medium (alone) or culture medium containing 200 μ Ci/ml ^{35}S -cysteine and 200 μ Ci/ml ^{35}S -methionine. After a 12 hour incubation, the conditioned medium is collected, concentrated on a spin filter, and loaded onto a 15% SDS gel. The processed gel may be dried and exposed to film for a selected period of time to reveal the presence of PRO polypeptide. The cultures containing transfected cells may undergo further incubation (in serum free medium) and the medium is tested in selected bioassays.

In an alternative technique, PRO polypeptide may be introduced into 293 cells transiently using the dextran sulfate method described by Somparyrac et al., *Proc. Natl. Acad. Sci.*, 12:7575 (1981). 293 cells are grown to maximal density in a spinner flask and 700 μ g pRK5-PRO polypeptide DNA is added. The cells are first concentrated from the spinner flask by centrifugation and washed with PBS. The DNA-dextran precipitate

is incubated on the cell pellet for four hours. The cells are treated with 20% glycerol for 90 seconds, washed with tissue culture medium, and re-introduced into the spinner flask containing tissue culture medium, 5 µg/ml bovine insulin and 0.1 µg/ml bovine transferrin. After about four days, the conditioned media is centrifuged and filtered to remove cells and debris. The sample containing expressed PRO polypeptide can then be concentrated and purified by any selected method, such as dialysis and/or column chromatography.

5 In another embodiment, PRO polypeptides can be expressed in CHO cells. The pRK5-PRO polypeptide can be transfected into CHO cells using known reagents such as CaPO₄ or DEAE-dextran. As described above, the cell cultures can be incubated, and the medium replaced with culture medium (alone) or medium containing a radiolabel such as ³⁵S-methionine. After determining the presence of PRO polypeptide, the culture medium may be replaced with serum free medium. Preferably, the cultures are incubated for about 6 days, and then the
10 conditioned medium is harvested. The medium containing the expressed PRO polypeptide can then be concentrated and purified by any selected method.

Epitope-tagged PRO polypeptide may also be expressed in host CHO cells. The PRO polypeptide may be subcloned out of the pRK5 vector. The subclone insert can undergo PCR to fuse in frame with a selected epitope tag such as a poly-his tag into a Baculovirus expression vector. The poly-his tagged PRO polypeptide
15 insert can then be subcloned into a SV40 driven vector containing a selection marker such as DHFR for selection of stable clones. Finally, the CHO cells can be transfected (as described above) with the SV40 driven vector. Labeling may be performed, as described above, to verify expression. The culture medium containing the expressed poly-His tagged PRO polypeptide can then be concentrated and purified by any selected method, such as by Ni²⁺-chelate affinity chromatography.

20 Stable expression in CHO cells was performed using the following procedure. The proteins were expressed as an IgG construct (immunoadhesin), in which the coding sequences for the soluble forms (e.g. extracellular domains) of the respective proteins were fused to an IgG1 constant region sequence containing the hinge, CH2 and CH2 domains and/or is a poly-His tagged form.

Following PCR amplification, the respective DNAs were subcloned in a CHO expression vector using
25 standard techniques as described in Ausubel et al., *Current Protocols of Molecular Biology*, Unit 3.16, John Wiley and Sons (1997). CHO expression vectors are constructed to have compatible restriction sites 5' and 3' of the DNA of interest to allow the convenient shuttling of cDNA's. The vector used expression in CHO cells is as described in Lucas *et al.*, *Nucl. Acids Res.* 24: 9 (1774-1779 (1996), and uses the SV40 early promoter/enhancer to drive expression of the cDNA of interest and dihydrofolate reductase (DHFR). DHFR
30 expression permits selection for stable maintenance of the plasmid following transfection.

Twelve micrograms of the desired plasmid DNA were introduced into approximately 10 million CHO cells using commercially available transfection reagents Superfect® (Quiagen), Dosper® or Fugene® (Boehringer Mannheim). The cells were grown and described in Lucas *et al.*, *supra*. Approximately 3 x 10⁻⁷ cells are frozen in an ampule for further growth and production as described below.

35 The ampules containing the plasmid DNA were thawed by placement into water bath and mixed by vortexing. The contents were pipetted into a centrifuge tube containing 10 mLs of media and centrifuged at 1000 rpm for 5 minutes. The supernatant was aspirated and the cells were resuspended in 10 mL of selective media (0.2 µm filtered PS20 with 5% 0.2 µm diafiltered fetal bovine serum). The cells were then aliquoted into a 100